Does MRF4 play a role in regulation of adult muscle phenotype?

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Forord

Denne masteroppgaven ble utført ved institutt for molekylær biovitenskap, program for fysiologi, under veiledning av professor Kristian Gundersen.

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Cecilie Sjåland

Abstract

Skeletal muscle fibre types are determined by the activity of motor neurons, hormones, stretch and probably cell lineage. The electrical activity of the motor neurons might, through an unknown pathway, influence the expression of a unique set of muscle specific genes in the different fibre types.

MRF4 is a member of a family of myogenic basic helix-loop-helix transcription factors (bHLH) that is important for the development of muscle fibres. These transcription factors are also suggested to be important in adult muscle, where they might be a possible link between electrical activity and expression of muscle specific genes. The myogenic bHLH transcription factors MyoD and myogenin has been shown to possible be involved in the regulation of fibre type specificity. This role is also proposed for MRF4, based on the high amount of MRF4 mRNA that is expressed in all adult skeletal muscle types and that it seems to be preferentially expressed in slow fibre types.

In this thesis I wanted to further examine if MRF4 might possible contribute in the regulation of fibre type specificity in normal adult skeletal muscles.

MRF4 was overexpressed in the fast glycolytic calf muscle; extensor digitorum longus (EDL). Plasmids containing the MRF4 gene and a reporter gene were transfected into the muscle by electroporation. After 14 days there was no change in the cross-sectional area or SDH activity in MRF4-transfected fibres compared to the control (sham)-transfected fibres. There were a significant higher number of 2b fibres in both MRF4- and sham-transfected fibres compared to normal, non-transfected fibres. This higher amount of 2b fibres is speculated to be caused by a possible selective transfection of the largest fibres as an effect of electroporation properties, or an effect of the lacZ itself or both.

These results indicate that MRF4 induces no change in MyHC isoforms, cross-sectional area and oxidative metabolism in normal adult skeletal muscle and this does not support the hypothesis that MRF4 may have a role in regulating fibre type specific patterns of muscle gene expression in adult skeletal muscle.

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1. Introduction

Skeletal muscles are composed of structurally, functionally and metabolically distinct fibre types. The different fibre types have the ability to change their size, composition of fibre types and enzymatic properties to accommodate altered functional demands. These alterations can occur because of altered electrical activity due to exercise, mechanical loading and unloading and hormones and aging, which all defines the muscle plasticity. This plasticity requires changes in the gene expression of muscle specific genes. There have been several studies on the pathways that link electrical activity to gene expression, and in this thesis I propose MRF4 to be a possible link between electrical activity and gene expression.

1.1. Fibre types

The fibre type diversity and transition abilities in skeletal muscles depend on the expression of the proteins that are involved in the excitation-contraction-relaxation cycle and their isoforms. The most common way to distinguish between the different fibre types is to look at the different myosine heavy chain isoforms (MyHCs) they express, and the different metabolic enzyme activity levels. Mammalian skeletal muscles express 11 MyHC isoforms and four of these are expressed in adult rodent skeletal muscle.

In the 19th century the fibre types was categorized as “red” with slow muscle contraction and “white” with fast muscle contraction. These have later been classified as type I, type IIa and type IIb fibres based on their ATPase activity and pH liability (Brooke & Kaiser, 1970). The ATPase activity varies by how fast the ATP is hydrolysed during the cross-bridge cycling in the exitation-contraction coupling. This variation in ATP activity has been shown to correlate with variation in contraction speed and MyHC isoform expression in the different fibre types (Reiser *et al.*, 1985)

With the use of methods like electrophoretic analysis of MyHC isoforms (Reiser *et al.*, 1985), mATPase histochemistry (Brooke & Kaiser, 1970) and immunohistochemistry using antibodies against the MyHC isoforms (Schiaffino *et al.*, 1989), the different MyHCs in skeletal muscle are now classified into four main groups: MyHCIβ, MyHCIIa, MyHCIIx and MyHCIIb.

The MyHCIβ represents the slow type I fibre type, with slow contraction speed and high oxidative capacity. The MyHCIIa, MyHCIIx and MyHCIIb represent the three fibre types; IIa, IIx and IIb in the fast type II population. Within the fast fibre population, the type IIa fibres have

fast contraction speed and have an oxidative-glycolytic metabolisme. The type IIb fibres have the fastest contraction speed and a glycolytic metabolism. The type IIx fibres are intermediates of these (Bottinelli *et al.*, 1991; Bottinelli *et al.*, 1994a; Bottinelli *et al.*, 1994b) see table 1.1. MyHCIIb has been shown not to be expressed in human or other large mammalian muscle (Smerdu *et al.*, 1994).

Fibre Type	Myosin Heavy Chain	Contraction Speed	Metabolic activity	Endurance
I	MHCIb	Slow	Oxidative	Good
IIa	MHCIIa	↓	Oxidative-glycolytic	Medium
IIx	MHCIIx			Poor
IIb	MHCIIb	Fast	Glycolytic	Very Poor

Table 1.1 Overview of the MHC contends and physical properties of the different fibre types.

1.2. Fibre type transitions

Muscle fibres have the capacity to alter their physical and functional properties to meet functional demands. These alterations can occur because of changed expression of the different proteins involved in the excitation-contraction-relaxation cycle due to altered nerve activity, mechanical loading and unloading, hormones and aging.

The nerve and electrical activity has an important influence on muscle contraction and fibre type composition (Gundersen, 1998). An alteration in the electrical activity induces a change in fibre type properties. In the absence of electrical activity by denervation, slow muscles become faster and fast muscles become slower (Huey & Bodine, 1998). Lomo et al, (1974) denervated the slow twitch soleus muscle and stimulated it with an electrical pattern normal for a fast twitch muscle, and saw that the slow soleus muscle attained several properties characteristic of a fast muscle. The opposite is true for stimulation of the fast EDL muscle with an electrical pattern normal for a slow twitch muscle; it will become more like a slow twitch muscle (Eken & Gundersen, 1988).

The conversion of a slow twitch muscle to a fast twitch muscle and a fast twitch muscle to a slow twitch muscle also seem to induce a change in myosin heavy chain composition and thus a change in fibre type composition.

Rat EDL consist of on average 45% type IIB, 29% type IIX, 23% type IIA and 3% type I fibres (Windisch *et al.*, 1998). Ausoni et al, (1990) showed that EDL muscles stimulated with a

slow pattern, showed a predominans of IIa-IIx type fibres, no fast type IIb-MyHC expression and a normal type I-MyHC expression whereas when the soleus muscle, which consist of on average 85 % type I fibres, 15 % IIa fibres, less than 1 % IIx fibres and no , IIb fibres (Rhana, master thesis 2003, Ausoni *et al*., 1990), was stimulated with a fast pattern, developed a predominans of IIa-IIx fibres, small amounts of type I-MyHC and no type IIb-MyHC (Ausoni *et al.*, 1990).

(Windisch *et al.*, 1998) showed that after stimulating the EDL muscle with a slow pattern for 4 months, all fibres reacted with the type 1 antibody, which indicate an almost complete transformation. This ability to an almost complete transformation in the EDL is also seen in the other direction in the soleus muscle. After 40 days of stimulation with a fast pattern the MyHCI isoform is redused to less than 35% and MyHCIIx becomes the prominent MyHC. MyHCIb increased to approximately 15% (Hamalainen & Pette, 1996).

Studies have also shown the existents of hybrid fibres. Hybrid fibres consist of two or sometimes more, MyHC isoforms (Pette & Staron, 1990; Schiaffino & Reggiani, 1996; Stephenson, 2001). The hybrid fibres are classified according to their MyHC isoform combinations: MyHCI+MyHCIIa (type I/IIa), MyHCIIa+MyHCIIx (type IIa/IIx), and MyHCIIx+MyHCIIb (type IIx/IIb). Hybrid fibres exist in normal fibres but are mostly common in fibres undergoing molecular and functional transitions:

Fast to slow conversion: IIb → IIb/IIx → IIx → IIx/IIa → IIa → IIa/I → I

Slow to fast conversion: I → I/IIa → IIa → IIa/IIx → IIx → IIx/IIb → IIb

During exercise training, muscles experiences an increased neuromuscular activity similar to what is seen when stimulating a muscle with chronic low-frequency stimulation, but to a lesser extent than with direct stimulation of the muscle. Exercise increases oxidative enzyme metabolism (Baldwin *et al.*, 1972; Holloszy & Booth, 1976) and in some cases induces fast-to-slow MyHC transitions, but this transition is limited to shifts in the slow direction in the fast fibre type population (Andersen and Henriksson, 1977). Extreme exercise might induce transitions from the fast fibre population to type I fibres (Demirel *et al.*, 1999).

Strech overload (Pattullo *et al.*, 1992), reduced levels of thyroid hormones (Fitts *et al.*, 1980) and aging (Larsson & Ansved, 1995) also induces a fast-to slow conversion in muscle phenotype.

1.3. Mechanisms involved in fibre type transitions

Skeletal muscle fibre types are, amongst other factors, determined by the activity of the motor neuron that innervates them and the unique set muscle specific genes the different fibre types express. These are well known facts, but the pathways that link the motor neuron activity with the expression of these muscle specific genes are still unclear. However, recent studies can now unravel several suggestions to what these pathways could look like.

A well studied pathway that is suggested to control skeletal muscle fibre types is the calcineurin dependent transcriptional pathway. Calcineurin can interact with several downstream substrates to activate gene transcription, and one of these is the nuclear factor of activated t-cells (NFAT) transcription factors. Calsineurin dephosphorylate NFAT so that NFAT can migrate to the nucleus and bind to transcriptional regulators.

Chin et al, (1998) found that the calcineurin dependent pathway influences fibre-type-specific gene expression in the fast to slow direction in cultured cells. They proposed a model where tonic motor nerve activity, characteristic of nerves that innervate slow muscles, sustains a Ca^{2+} level suffient to activate the calcineurine-NFAT pathway. Fast fibres, with infrequent, phasic firing of the motor nerve, were of insufficient duration to maintain calcineurin activation. They also found that NFAT can bind to the Mef-2 transcription regulator which is important in activating the myogenic bHLH factors. The role of the calsineurin-NFAT pathway has also been proven to have the same effect in adult skeletal muscle in vivo (McCullagh *et al.*, 2004).

Calcineurin can also act through factors other than NFAT to promote the slow fibre type program. Calcineurin dependent gene regulation in skeletal myocytes is also mediated by Mef-2 either through NFAT or in the absence of NFAT. Mef-2 can also be stimulated in the absence of calcineurin but in the presence of calmodulin-dependent protein kinase activity, but the effect on Mef-2 is greatly enhanced in the presence of both calcineurin and CaMK (Wu *et al.*, 2000).

Another pathway involved in promoting nerve-activity-dependent differentiation of slow muscle fibres *in vivo* is the Ras-MAPK pathway. Activated Ras induces the expression of slow MyHC (Murgia *et al.*, 2000).

Myogenin is a bHLH transcription factor that is mostly expressed in slow fibre types (Voytik *et al.*, 1993), and myogenin seems to mediate transcription of oxidative genes and is proposed to be linked to slow motor neuron activity via the calcineurin pathway (Ekmark *et al.*, 2003).

PPARδ is a transcription factor that also seems to mediate expression of slow fibre type genes. PPARδ is activated by free fatty acids, and slow motor neuron activity due to exercise is known to up-regulate this ligand. PPARδ is also known to be activated by PGC-1 which is a co-factor that physically associates with PPARδ in muscle tissue and can strongly activate PPARδ even in the absence of ligands (Wang *et al.*, 2004).

Slow-to-fast fibre transformation occurs as a consequence of decreased motor neuron activity, due to cross-innervation, certain disease states or physical inactivity. MyoD is a bHLH factor that is highly expressed in fast fibre types (Voytik *et al.*, 1993) and is proposed to be linked to a pathway mediating fast motor neuron activity to fast fibre gene transcription (Ekmark, unpublished). Possible signalling pathways and cross-reactions are shown in figure 1.1

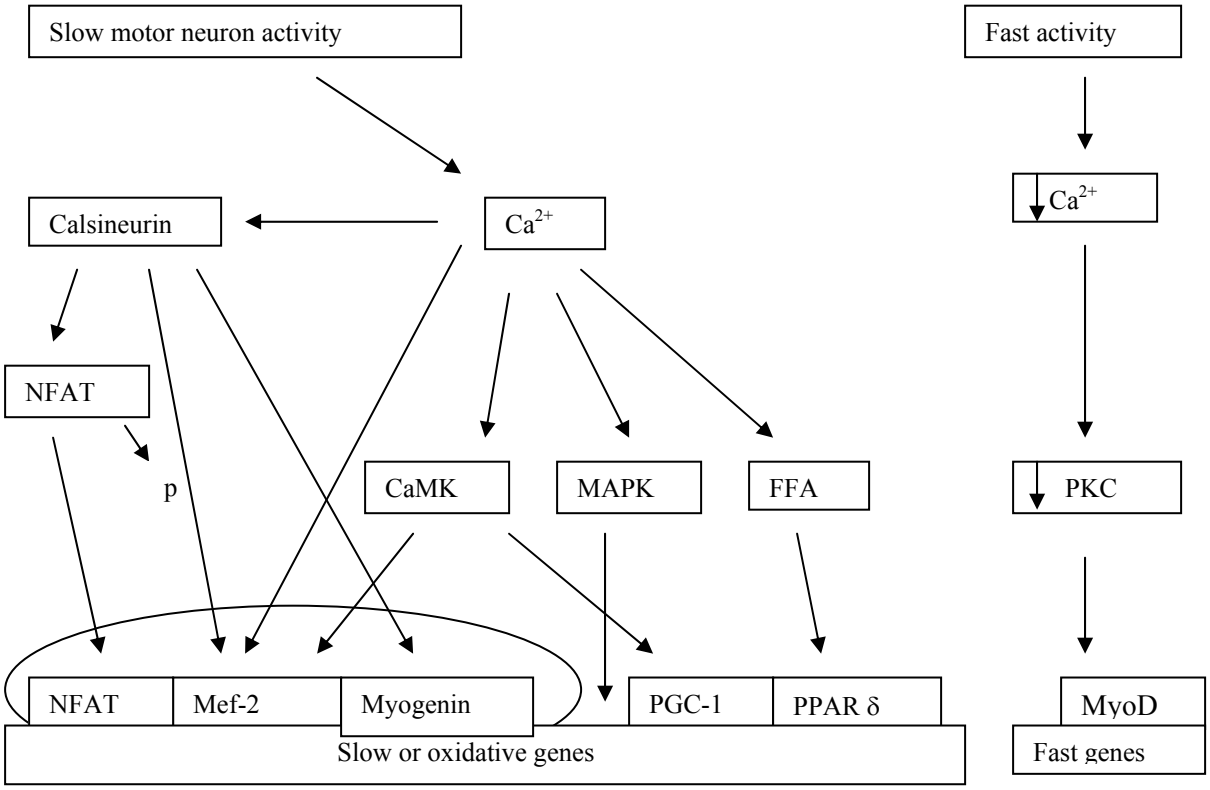


Figure 1.1 Overiew of signalling pathways that might link electrical activity to transcription factors and gene expression.

1.4. Myogenic determination factors

The myogenic determination factors are a group of transcription factors that belongs to a family of conserved basic helix-loop-helix transcription factors (bHLH). These myogenic determination factors are known to have an important function in the development of somite cells into muscle cells, known as the myogenesis.

The myogenic bHLH proteins can form heterodimers with the HLH protein E12 which binds the consensus DNA E-box CANNTG sequence (figure 1.2). E-box sequences are found in both promotor and enhancer areas of muscle specific genes (Olson, 1990; Buonanno *et al.*, 1992; Emerson, 1993; Weintraub, 1993).

Other studies have shown that members of the myocyte enhancer factor-2 (MEF-2) family of MADS-box transcription factors are important in the myogenesis. The MEF-2 binding site has been identified in control regions of almost all skeletal muscle genes. These factors cooperate with myogenic bHLH factors to activate muscle gene expression. This indicates that activation signals from both the MEF-2 and the myogenic bHLH factors are required for initiation of the myogenesis (Reviewed by (Black & Olson, 1998)

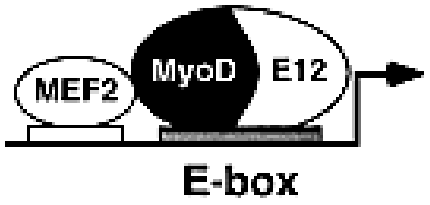


Figure 1.2 Potential mechanisms for activation of skeletal muscle transcription by MEF2 and myogenic bHLH factors (Black & Olson, 1998)

1.5. Myogenesis

The myogenesis is the formation of somite progenitor cells into myoblasts, myocytes and myotubes. Further differentiation of the myotubes forms the skeletal muscle fibres.

The formation of skeletal muscle fibres are driven by the expression of four myogenic determination factors MyoD, Myf- 5, myogenin and MRF4 (Davis *et al.*, 1987; Braun *et al.*, 1989; Edmondson & Olson, 1989; Rhodes & Konieczny, 1989; Wright *et al.*, 1989; Braun *et al.*, 1990; Miner & Wold, 1990).

The four myogenic factors are expressed at different stages during the myogenesis; Myf-5 transcripts can be seen at E8 as the segmentation of the somites begins. The myogenin mRNA is detectable at E8.5 when myotomal muscle begins to form. The MRF4 mRNA is expressed transiently at E9 and only in the myotomes. After E16 the MRF4 mRNA is expressed in all skeletal muscles and becomes the dominant transcription factor in adult skeletal muscle. MyoD mRNA can be seen at E10.5 and the expression persists throughout the prenatal life (Sassoon *et al.*, 1989; Bober *et al.*, 1991; Hinterberger *et al.*, 1991; Ott *et al.*, 1991; Arnold & Braun, 1996).

Because the myogenic factors are expressed at different times and at different levels during the myogenesis, it indicates that they have different functions throughout the myogenesis. To investigate the role of the myogenic factors during myogenesis, several studies on inactivation of the myogenic factor genes have been conducted.

Mice lacking the functional MyoD gene are viable and fertile, and exhibit no morphological or physiological abnormalities. All myogenic mRNA is expressed at normal levels except that of Myf-5 which is increased 3.5-fold, suggesting that normal Myf-5 expression is inhibited by MyoD, or that Myf-5 compensates for the lack of MyoD (Rudnicki *et al.*, 1992).

Mice lacking Myf-5 showed normal development of skeletal muscle but died immediately after birth because of major rib abnormalities that impedes breathing (Braun *et al.*, 1992).

To further explore the roles of Myf-5 and MyoD in the myogenesis, mice lacking both MyoD and Myf-5 were investigated. These mice were born alive but were immobile and died soon after birth. There was no formation of skeletal muscle which suggests that either Myf-5 or MyoD is required for the determination of skeletal myoblast, their proliferation or both (Rudnicki *et al.*, 1993).

Mice lacking the myogenin gene die immediately after birth and show severe reduction of all skeletal muscle. This suggests that myogenin plays an important role in the differentiation of myoblasts into myotubes. The MRF4 mRNA level is decreased, indicating that myogenin might be necessary for normal MRF4 levels (Hasty *et al.*, 1993).

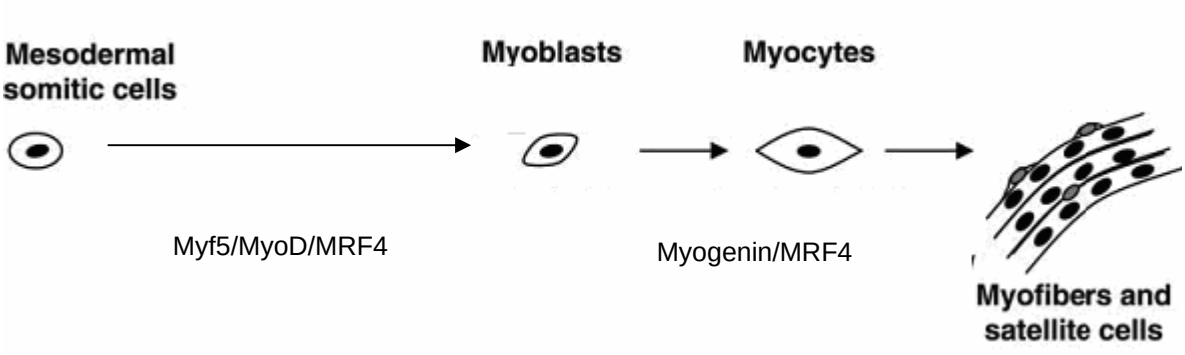
Mice lacking the MRF4 gene were viable and showed no abnormalities in the muscle formation, but there were defects in the rib development. No other skeletal defects were observed. There was an up-regulation of myogenin mRNA that suggests that MRF4 normally

represses myogenin expression after birth and might compensate for the absence of MRF4 (Zhang *et al.*, 1995).

These results taken together suggests that Myf-5 and MyoD are primary myogenic determination factors that are important for the development of somatic cells into myoblasts, and that myogenin and MRF4 are secondary determination factors that are important for the differentiation of myoblasts into myocytes and myotubes.

The role of MRF4 as a secondary determination factor has been the general conception, but a resent study has shown that MRF4 also might be one of the early determination factors. A study by Kassar-Duchossoy, (2004) indicates that MRF4 also contributes to the determination of skeletal myoblast (figure 1.3)

The results also indicate that these myogenic factors are involved in a cross-regulatory network in which the activity of one factor regulates its own and other myogenic bHLH gene's expression.



Figur 1.3 Myf-5, MyoD and possible MRF4 determine the formation of somatic cells into myogenic cells. Myogenin and MRF4 trigger the formation of myoblast into myotubes and myofibres. (Modified figure from (Charge & Rudnicki, 2004)

1.6. MDF's in adult muscles

The myogenic factors have proven to play an important role during the myogenesis, but they are also continued to be expressed in the adult muscle, suggesting that they might be important in modulating gene expression in adult muscles and thereby contribute to the diversity of individual muscle phenotypes and muscle plasticity.

Myogenin and MyoD are expressed in different amounts in slow and fast muscles. Myogenin is expressed in high levels in muscles with a high amount of slow fibre types, and in

low levels in muscles with high amounts of fast fibre types. MyoD is expressed in high levels in muscles with a high amount of fast fibre types and in low levels in muscles with a high amount of slow fibres (Hughes *et al.*, 1993; Voytik *et al.*, 1993).

MRF4 and Myf-5 is expressed at in both fast and slow muscles; MRF4 is expressed at equal high levels in all muscle types, whereas Myf-5 is expressed at equal low levels in all muscle types (Hughes *et al.*, 1993; Voytik *et al.*, 1993).

1.7. Myogenic bHLH factors and fibre type specificity

Based on the different distribution of the myogenic bHLH factors between different muscle types, there is reason to believe that these factors contribute to muscle fibre type specificity. Hughes et al, (1993) showed that MyoD and myogenin mRNAs accumulate in muscles that differ in contractile properties. MyoD accumulates in fast twitch muscles and myogenin accumulates in slow twitch muscles. Huges et al, (1993) also found that thyroid hormone treatment resulted in activation of both MyoD and fast MyHC gene expression in the slow soleus, and that cross-reinnervation of the soleus with a fast nerve results in decreased myogenin mRNA expression in regions now induced to express fast MyHC.

Hughes et al, (1997) studied the fibre type composition in MyoD null mice. In the absence of MyoD, there was a shift from a fast to a slower phenotype in fast muscles. In contrast, slow muscle shifts toward a faster phenotype.

In a current studie, the MyoD gene has been overexpressed in the slow soleus muscle, and immunohistological analysis shows an increase in fibre types that expresses the fast MyHCs, which indicates a shift in the slow-to-fast direction (Ekmark,unpublished data)

Myogenin also show the ability to change muscle phenotype. Overexpression of myogenin in transgenic mice showed a 2-3 fold elevation of oxidative mitochondrial enzymes in fast muscles and a reduced level of glycolytic enzymes. This revealed a myogenin induced shift in enzyme activity from glycolytic to oxidative metabolisme (Hughes *et al.*, 1999). Overexpression of myogenin in pre-existing adult fibres in the fast EDL muscle, showed an increase in oxidative enzyme levels and a reduction in the fibre cross-sectional area, without changes in the major MyHC fibre type (Ekmark *et al.*, 2003). This indicates a change in the slow direction similar to the change in muscle fibres during endurance training.

1.8. MRF4- A role in regulation of fibre phenotype in adult skeletal muscle?

MRF4 mRNA is expressed in high amounts in both fast and slow muscles (Voytik *et al.*, 1993), but Walters et al, (2000) found that in a mixed muscle, such as the gastrocnemius muscle, there was a higher expression of MRF4 in the deeper parts which consists of mostly type I fibres, than in the surface part of the muscle which consists mostly of type II fibres. Based of these findings they suggested that MRF4 might exhibit a fibre type specific pattern of expression in adult muscles.

The bHLH myogenic factors have been suggested to be involved in regulation of fibre type specific patterns in adult muscles. MyoD has been suggested to have a role in regulation and maintaining a fast fibre type pattern and myogenin has been suggested to be involved in the regulation of oxidative capacity and fibre size(strength) in the slow direction (Hughes *et al.*, 1997; Ekmark *et al.*, 2003).

Both MRF4 and myogenin expression is regulated by the binding of their subunit to the consensus DNA E-box CANNTG sequence. E box sequences are found in both promotor and enhancer areas of muscle specific genes (Olson, 1990; Buonanno *et al.*, 1992; Emerson, 1993; Weintraub, 1993). MRF4 and myogenin also bind to Mef-2 which is known to activate transcription of muscle specific genes (Black & Olson, 1998). Studies with knock out mice suggest that myogenin has a compensatory role in the absence of MRF4 and that myogenin is necessary for normal levels of MRF4 (Hasty *et al.*, 1993; Zhang *et al.*, 1995).

These studies suggest that MRF4 might have a role similar to that of MyoD and myogenin in regulating fibre type specific patterns in adult skeletal muscle and then possible in the slow direction, like myogenin.

1.9. Aim of the study

Will overexpression of MRF4 in adult skeletal muscle induce changes in fibre type composition, oxidative capacity and fibre cross-sectional area?

MRF4 was transfected into muscle fibres in the fast EDL muscle by electroporation, to see if MRF4 might induce changes in MyHC isoforms, cross-sectional area and oxidative capacity to a pattern normal in slow fibre types.

2. Material and Methods

2.1. Overwiev

The Extensor digitorum longus (EDL) in rat was surgically exposed and transfected with plasmids containing either the MRF4 gene and reporter gene lacZ or just the reporter gene as a control. The plasmids were transfected by electroporation. After 14 days, the electroporated EDL muscles were removed and freezed and cross sections were prepared. Histological analyses, like staining for β- gal, SDH and myosin heavy chains (MyCH), were conducted on neighbouring sections (figure 2.1)

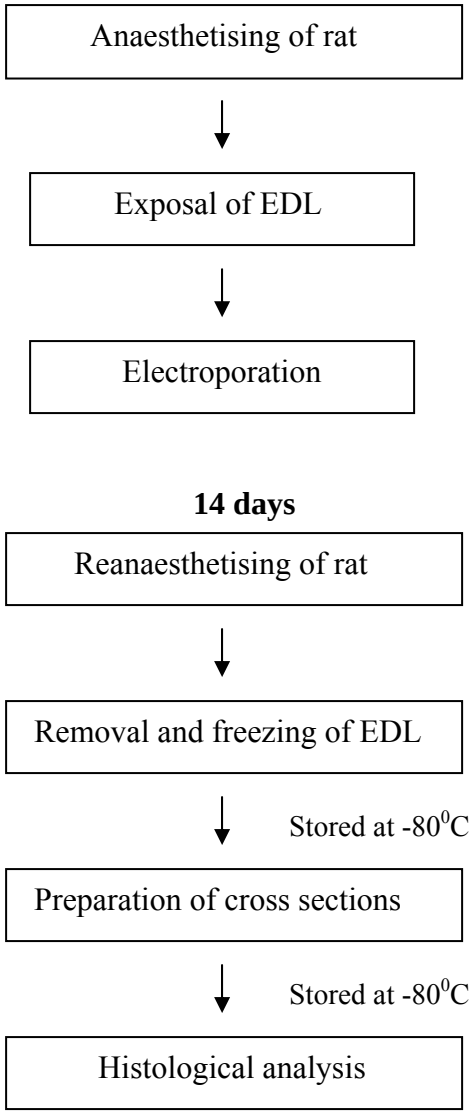


Figure 2.1 Overview of the experimental methods

2.2. Animal experiments

The experiments were performed on male wistar rats (200-400g), delivered by the Norwegian institute of public health, and kept in animal research facilities at the University of Oslo. The animals were kept in cages in rooms with a regulated temperature at 22°C and with air humidity at 50-60%. Food and water were given ad libitum.

The animals were anesthestetised by interperitoneal injections with Equthisin (5µl/g) (appendix A, 5.1) and the effect of the anesthetics were controlled by pinching the metatarsian region of the rat leg to check for absents of reflex. If necessary, extra anesthesia was administrated. When the animal were under deep anesthesia, hair were first removed by shaving the leg with a machine and followed by applying hear removal cream (Veet for sensitive skin, Reckitt and Coleman). The rat was laid on a cork plate and the leg was fixed on to a styrofoam bloc by pinning it in a locked position between the toes. The extensor digitorium longus (EDL) muscle was exposed surgically and DNA was injected and then transfected into the muscle cells by electroporation. The wound was surgically closed with sutures.

All animal experiments were approved by the Norwegian Animal Research Authority and were conducted in accordance to the Norwegian Animal Welfare Act of December 20th, 1974, no. 37, chapter VI, sections 20-22, and the Regulation on Animal Experimentation of January 15th 1996.

2.3. Plasmids

The rat EDL muscle was injected with a DNA solution containing a mix of the plasmids pAP-MRF4 (figure 2.2B) and pAP-lacZ in the right muscle. Studies have shown that two plasmids that are coinjected will be coexpressed after transfection (Rana et al, 2004). The left EDL muscle was injected only with the pAP-lacZ plasmid (figure 2.2A) as a control.

The pAP-lacZ plasmid (Kisselev *et al.*, 1995) contain the Escherichia Coli β-galactosidase coding sequence driven by the Rouse sarcoma virus (RSV) promoter and an origin of replication driven by a SV40 promotor.

The construction of pAP-MRF4 was made by first cloning the isolated MRF4 PCR product in to an Easy vector (promega, protocol.) and further cloned into a pGEFP vector by cutting the Easy vector with EcorI. From the pGEFP vector, the MRF4 was cloned in to the pAP –vector, made by removing the β-galactosidase coding sequence by cutting it with BamHI and

HindIII. The MRF4 was cloned into the pAP-vector after cutting the multiple restriction sites in pGEFP with BamHI and HindIII for directional insertion. The pAp-MRF4 plasmid now contains a MRF4 coding sequence driven by the RSV promoter and an origin of replication driven by the SV40 promotor.

A **B**

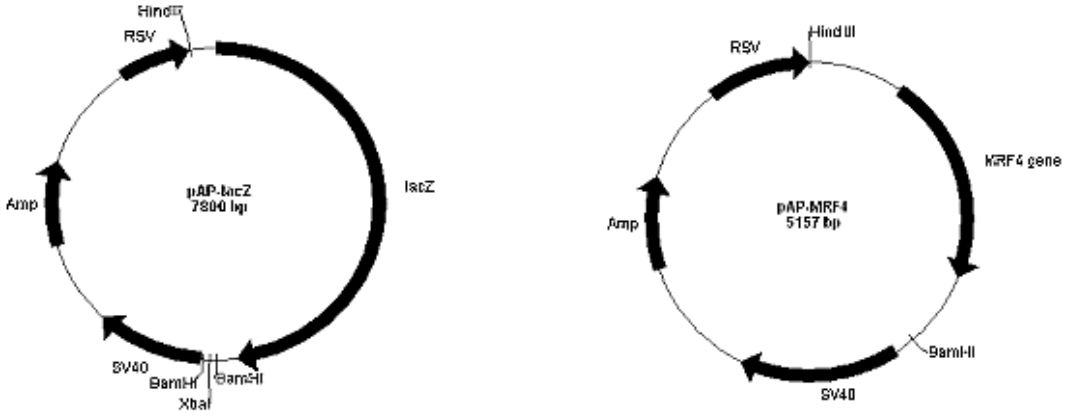


Figure 2.2 Plasmids.
A. The plasmid pAP-lacZ gene which expresses the β-galactosidase protein. **B.** The plasmid pAP-MRF4 contains the MRF4 gene which expresses the MRF4 protein

Experiment groups	Plasmids	Overexpressed protein
Normal	-	-
Control	pAP-lacZ	β-galactosidase
MRF4	pAP-MRF4	MRF4

Table 2.1 Overview of the experiment groups

2.4. Transfection of plasmids

2.4.1. Transfection in Tissue Culture

To test for expression of MRF4, human embryonic kidney cells (HEK-239) were transfected with pAP MRF4 and pAP-lacZ as a control, using the Lipofectamine2000 kit (Invitrogen). The MRF4 protein were extracted from these cells and visualized by western blotting (figure 2.3). The MRF4 protein was visualized by first applying a specific MRF 4 antibody, kindly donated by Andres Buannano, and then by applying a secondary goat anti mouse IgG antibody (Southern Biotech).

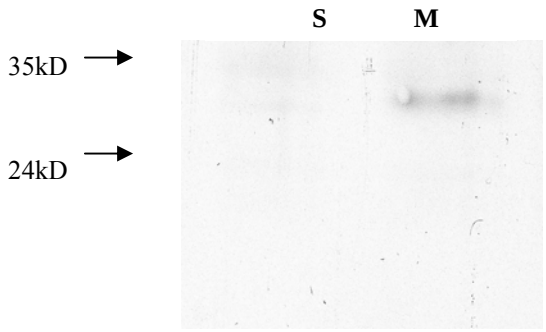
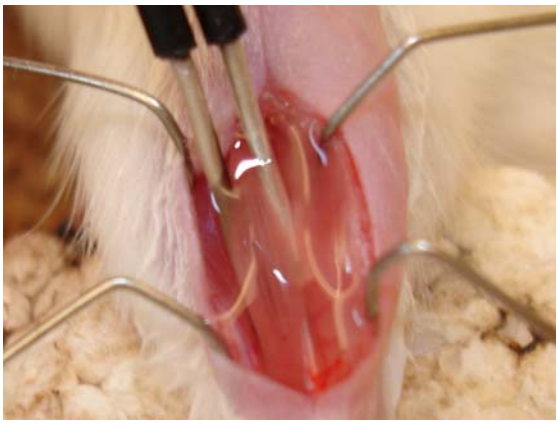


Figure 2.3 Expression of MRF4 in tissue culture. Western blot of protein extracts from human embryonic kidney cells transfected with the sham (S) plasmid pAP-lacZ or the MRF4 (M) overexpressing plasmid pAP-MRF4.

2.4.2. Electroporation

Transfection of the DNA was performed *in vivo* as described previously by Mathiesen (1999). The Extensor digitorium longus (EDL) muscle was exposed surgically and 100µl DNA (appendix A, 5.2 and 5.3) was injected through a Hamilton syringe to the centre of the muscle. The muscle was then subjected to an electrical field by a pulse generator (Pulsar 6bp-a/s, Fredrick Haer & Co). Two silver electrodes, 1mm thick, placed ca 3mm a part were moved along the muscle and five trains of bipolar pulses (200µs long in each direction) with a peak to peak

voltage of 50V were run across the muscle (figure 2.4). The electrical charge was registered with an analogue oscilloscope (03245A, Gould Advance).



Figur 2.4 Electroporation
To silver electrodes on each side of the EDL muscle, which has been injected and baded in DNA solution, creates an electrical charge across the muscle.

2.5. Histology

2.5.1. Freezing of EDL

The EDL muscle was surgically removed 14 days after transfection of the DNA. The muscle was lightly stretched between two pins on a double layer of thin wax blocks. The muscle was then lowered into a container with liquid isopentane which was cooled down to the freezing point at ca -160°C with liquid nitrogen. After frozen in isopentane, the muscle was lowered into liquid nitrogen before contained in cooled cryotubes (Nunc Cryoflex ^{TM} Tubling) and stored at -80 °C.

2.5.2. Preparation of cryosections

The muscle was fastened to a holder by applying O.C.T Compound (Tissue Tek) and then mounted in the cryotome (HM560M Microme). The muscle tissue was kept at a temperature of -18°C and the cutting knife was kept at a temperature of -24°C. 10µm serial sections was made and placed on microscope slides and stored at -80°C for further analysis.

2.5.3. β-gal staining

β-gal is the reporter protein used in this experiment. Staining for β-gal activity will identify the transfected fibres. β-gal is determined histochemically (appendix A, 5.4) by adding 5-brom-4chloride-3indoloyle-β-D-galactoside (x-gal) to the sections. β-gal will hydrolyze to the colourless product indoxyle. Indoxyle dimerises and gives blue coloured crystals (figure 2.5). The crystals do not solve in water so the colour becomes permanent after mounted in glycerine gel. Staining without x-gal as substrate gave no blue fibres.

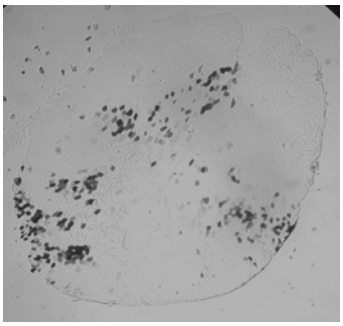


Figure 2.5 Staining for β-gal
The picture shows a cross section of the EDL muscle stained for β-gal. The fibres transfected with lacZ is here shown in black.

2.5.4. SDH staining

SDH is an enzyme localized in the mitochondria which catalyses the oxidation of succinate to fumarate in the citric acid cycle. The oxidative capacity of a muscle can be measured by the level of SDH activity in the muscle fibres. The determination of the SDH activity was performed as described by Bancroft (1975). SDH catalyses the reduction and protonation of tetrazolium to formazan, when succinate is used as a substrate. Tetrazolium is colourless and non-solvent in water while formazan is a crystalloid and projects a blue-purple colour (appendix A, 5.5). The colour stays permanent when mounted in glycerine gel. Staining without succinate as substrate gave no staining.

2.5.5. Staining for Myosine heavy chain isoforms

Monoclonal antibodies against MyHC I, MyHCIIa, MyHCIIb and all MyCHII (kindly provided by Stefano Schiaffino) were used to determine muscle fibre types. For visualizing the specific binding of primary antibodies, secondary antibodies were conjugated to the reporter

protein rhodamin and applied to the sections (table 2.2, appendix A 5.6). When rhodamin is looked at through green light, it will appear red. The fibres with the specific antibodies can then be visualized and identified. Staining without the primary antibodies gave no fluorescence for any of the primary antibodies.

MyHC	Primary antibody	Secondary antibody
I	BA-DF	Anti-Mouse Ig, Rhodamine (1214608, Boeheringer)
IIa	SC-71	Anti-Mouse Ig, Rhodamine (1214608, Boeheringer)
All II	MY-32	Anti-Mouse Ig, Rhodamine (1214608, Boeheringer)
IIb	BF-F3	Anti-mouse IgM, Cy-3 (J115-165-020, Jackson ImmunoResearch Laboratories)

Table 2.2 Overview of the antibodies used for staining for MyHC isoforms.

2.6. Imaging

2.6.1. Fluorescence imaging

Pictures of sections marked with antibodies conjugated to rhodamin were taken by a SIT video camera (C2400-08, Hamatsu). The camera was connected to a microscope (BX50WI, Olympus) with 10x and 20x water immersion objectives (UMPlan FI, Olympus). The filter cube XF33 (Omega Optical) was used to give a green light and select for the red light emitted from rhodamin.

2.6.2. Bright Field Imaging

Bright field pictures of sections stained for β-gal and SDH activity were taken by a CCD video camera (C2400, Hamatsu). The camera was connected to a microscope (BX50W1, Olympus) with 10x and 20x water immersion objectives (UMFPlanF1, Olympus). The images were digitalized through an image-processing unit (Argus-20, Hamatsu) before they were transferred to a Power Macintosh G3 computer.

2.7. Quantitative Histochemistry

2.7.1. SDH activity

SDH activity was measured in fibres that overexpressed MRF4, control transfected fibres and in normal fibers. SDH activity was determined by measuring the colour intensity in ImageJ (NIH). The fibres of interest were manually encircled and the mean pixel grey value was measured from a scale from 0 (white) to 253 (black). The scale was standardised for all individual sections to be able to compare the individual sections.

2.8. Calculation of cross sectional area

As encircling the fibres to measure SDH activity, the mean cross sectional area were also measured. The area was measured as number of pixels and was later converted to μm by calibrating against a μm scale.

2.9. Statistical Methods

An unpaired t-test and Anova with a Bonferroni post-test was used for statistic comparison of enzyme activity and cross-section area between the experiment groups.The level of significance was chosen to 5%.

A Fisher's exact test was used for statistic comparison of number of fibres transfected between MRF-, sham- and non-transfected fibres. Fisher's exact test calculates 2X2 contingency tables where the values are numbers of observations, and calculates the probability that each of these tables would have been obtained by chance.

The statistical calculations were performed in Graphpad Prism.

3. Results

3.1. Overview

Fibre type, cross-sectional area and SDH activity was identified and quantified in EDL muscles electroporated with either pAP-lacZ or both pAP-MRF4 and pAP-lacZ. Fibres electroporated with pAP-lacZ, which express the β-gal protein, are described as control transfected fibres, or sham fibres. Fibres electroporated with both pAP-lacZ and pAP-MRF4 are described as MRF4 transfected fibres, and is considered to overexpress MRF4. The transfected fibres were identified by staining for β-gal activity, as the fibres positive for β-gal activity appears as blue fibres. A number of non-transfected fibres were randomly picked in the near vicinity of the transfected fibres in both MRF4-transfected muscles and sham-transfected muscles. These are described as MRF4-normal fibres and sham-normal fibres and acts as an additional control within each muscle. Neighbour sections were stained for β-gal activity, SDH activity and MyHC isoforms and compared for analysis (figure 3.1).

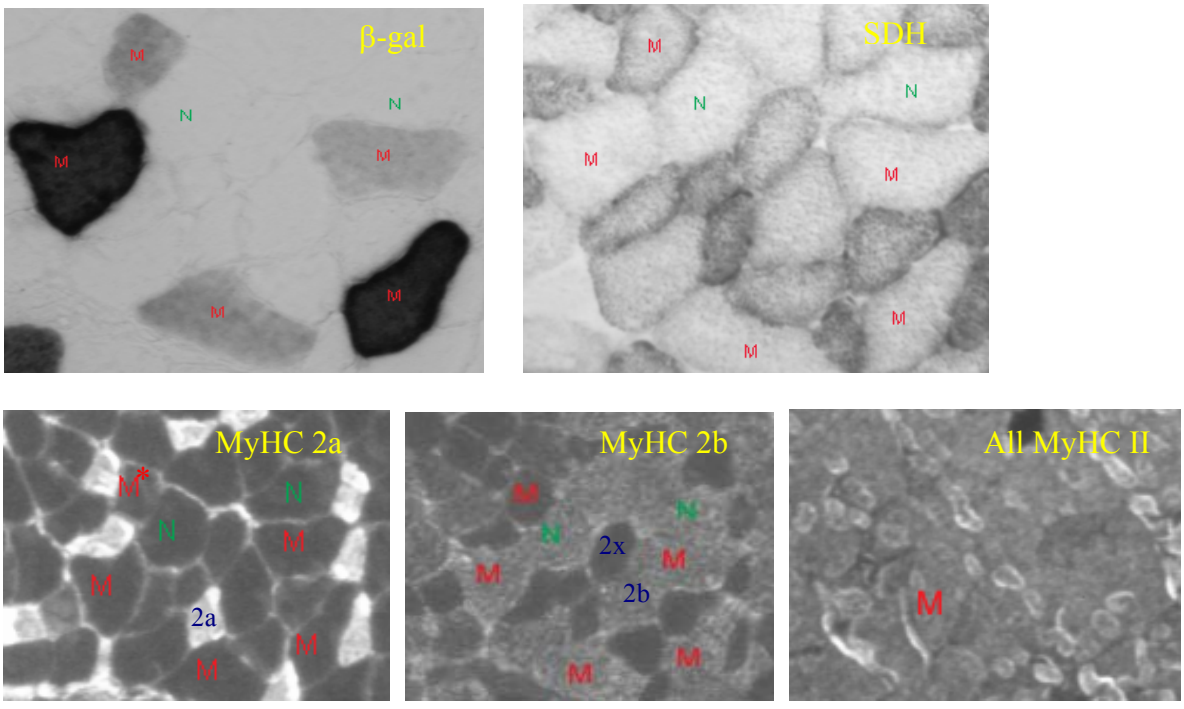


Figure 3.1 Cross sections of the EDL muscle electroporated with pAP-lacZ and pAP-MRF4.
The cross sections are stained for β-gal activity, SDH activity, MyHC IIa, MyHC IIb and all MyHC II. β-gal positive fibres are shown as black or grey fibres. The difference in the strength of the SDH staining is a measure of the difference in SDH activity. Stronger staining means higher SDH activity. The transfected fibres are marked M (MRF4). Non-transfected fibres or normal fibres are marked N. Looking at the MyHC staining, one can see that all the M and N marked fibres are all type 2b fibres except the M* which in this case is a 2x fibre. Exsamples of 2a, 2x and 2b fibres is marked on the pictures.

3.2. Fibre Types

The distribution of fibre types were identified by counting the number of 2a, 2x and 2b fibres in MRF4-transfected fibres, MRF4-normal fibres, sham-transfected fibres and sham-normal fibres (table 3.1).

The normal distribution of fibre types in normal EDL muscles is on average 45% type IIB, 29% type IIX, 23% type IIA and 3% type I fibres (Windisch et al., 1998). This coincide with the fibre type distribution in the MRF4-normal and sham-normal fibres in this study (table 3.1, figure 3.2) (because of only very few observed type I fibres, I have not included these in the results).

Because the total number of transfected fibres and the number of transfected 2a, 2x and 2b fibres varies between the individual muscles (table 3.2), the fibre type distribution is analysed as percentage of the total number of fibres in each experiment group.

There is a significant higher number of 2b fibres in both the MRF4-transfected fibres and the sham-transfected fibres ($p<0.0001$) and significant fewer 2a and 2x fibres ($p<0.0001$, $p=0.0030$) compared to the normal fibres. The results also show a significant higher number of 2b of sham-transfected fibres ($p=0.0001$) and subsequent significant fewer 2a and 2x fibres in sham-transfected fibres ($p=0.0176$, $p=0.0066$) compared to the MRF4-transfected fibres. There is no significant difference in number of 2a, 2x and 2b fibres between MRF4-normal and sham normal fibres (table 3.1, figure 3.2)

Comparing the number of 2b fibres in the MRF4-transfected muscles with sham transfected muscles, it looks as if there has been a transformation of MyHC isoforms in the slow direction, but if we compare both the MRF4- and sham transfected fibres with the normal fibres, this effect is not seen. The higher amount of 2b fibres in both MRF4- and sham-transfected fibres is speculated to be caused by a possible selective transfection of the largest fibres as an effect of electroporation properties, or an effect of the lacZ itself or both.

Fibre types	2a	2x	2b	
Experiment group	n (%)	n (%)	n (%)	Total
MRF4	30 (7,6) *	80 (20,2) *	286 (72,2) *	396
Sham	13 (3,5) *	48 (12,8) *	314 (83,7) *§	375
MRF4-normal	78 (19,6)	117 (29,4)	202 (51,0	397
Sham-normal	71 (19,2)	118 (32,0)	180 (48,8)	369

Table 3.1 Number and percentage of 2a, 2x, 2b fibres in the different experiment groups.
*There are a significant higher number of 2b fibres and significant fewer 2a and 2x fibres in MRF4- and sham-transfected fibres compared to the normal distribution. § There are a significant higher number of 2b fibres in sham-transfected fibres compared to MRF4-transfected fibres. There is no significant difference in the fibre type distribution between the MRF4- and sham-normal fibres.

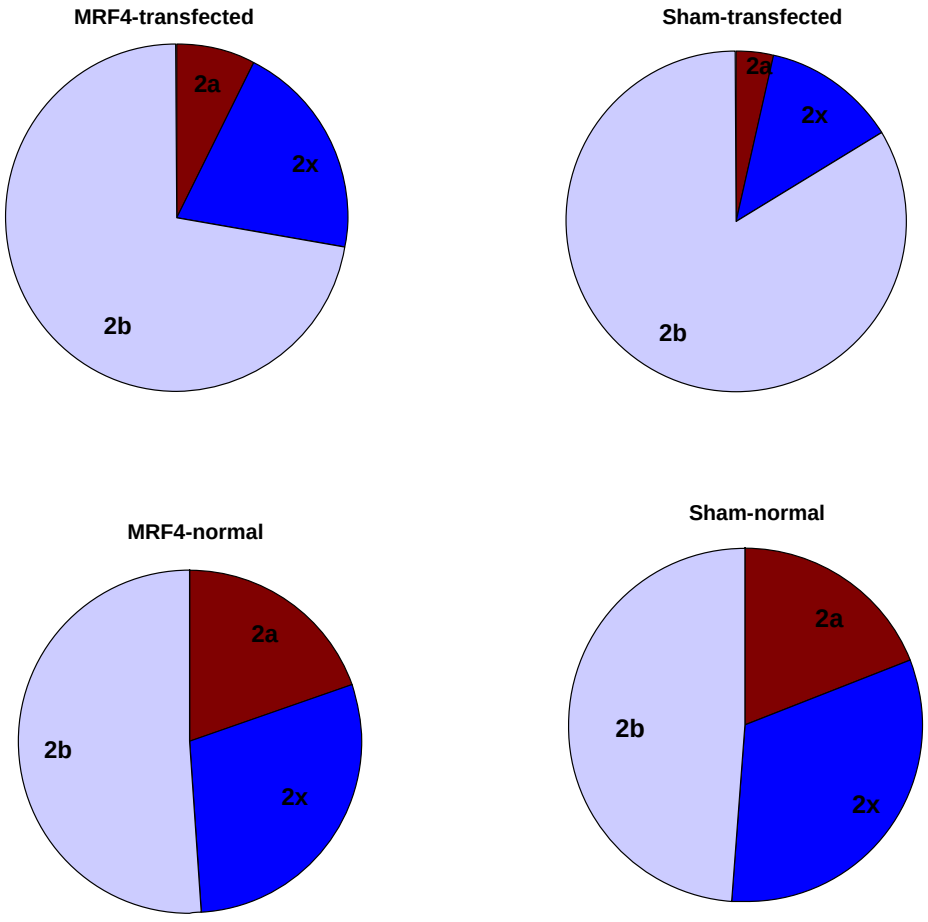


Figure 3.2 Pai charts showing the fibre type distribution in percentage of the total number of fibres in each experiment group.
There are a significant higher number of 2b fibres in both the sham -and MRF4-transfected fibres compared to MRF4-normal and sham-normal fibres. There is significant fewer 2a and 2x fibres in the sham -and MRF4-transfected fibres. There are a significant higher number of 2b fibres in the sham-transfected fibres compared to the MRF4-transfected fibres. There is no significant difference in the number of 2a, 2x and 2b fibres between MRF4-normal fibres and sham-normal fibres.

	Fibre types	2a	2x	2b	
Rat	Experiment group	n (%)	n (%)	n (%)	Total
A	MRF4	1 (4,3)	3 (13,1)	19 (82,6)	23
	Sham	2 (3,7)	7 (13,0)	45 (83,3)	54
	MRF4-normal	6 (26,1)	4 (17,4)	13 (56,6)	23
	Sham-normal	12 (22,6)	17 (32,1)	24 (45,3)	53
B	MRF4	2 (2,6)	11 (14,5)	63 (82,9)	76
	Sham*	NA	NA	NA	
	MRF4-normal	23 (30,3)	20 (26,3)	33 (43,4)	76
	Sham-normal*	NA	NA	NA	
C	MRF4	5 (6,2)	26 (32,1)	50 (61,7)	81
	Sham	9 (9,7)	21 (22,6)	63 (67,7	93
	MRF4-normal	8 (9,9)	25 (30,9)	48 (59,3)	81
	Sham-normal	17 (19,1)	32 (35,6)	40 (44,9)	89
D	MRF4	13 (11,5)	28 (24,8)	72 (63,7)	113
	Sham	0 (0)	4 (8,0)	46 (92,0)	50
	MRF4-normal	34 (29,8)	40 (35,1)	40 (35,1)	114
	Sham-normal	8 (16,0)	16 (32,0)	26 (52,0)	50
E	MRF4	9 (8,7)	12 (11,7)	82 (79,6)	103
	Sham	2 (1,1)	16 (9,0)	160 (89,9)	178
	MRF4-normal	7 (6,8)	28 (27,2)	68 (66,0)	103
	Sham-normal	34 (19,2)	53 (29,9)	90 (50,8)	177

Table 3.2 Number and percentage of 2a, 2x and 2b fibres within the experiment groups in the different individes.
The total number of transfected fibres and the number of transfected 2a, 2x and 2b fibres varies in the animals and the individual muscles. The percentage distribution of the fibre types in each animal show however an overall higher number in 2b fibres in sham-transfected fibres compared to MRF4-transfected fibres.

3.3. Cross-sectional Area

The cross-sectional area was measured in the transfected and normal fibres in all four experiment groups. In normal fibres, the 2a fibres have the lowest cross-sectional area while the 2b fibres have the highest. The 2x fibres have an intermediate cross-sectional area of these (figure 3.3).

Analysis of the average cross-sectional area of all muscles taken together, show a significant difference in the cross-sectional area in the 2a, 2x and 2b fibres of the MRF4-transfected fibres compared to sham-transfected fibres and in the MRF4-normal fibres compared to sham-normal fibres, but not in the MRF4-transfected fibres compared to MRF4-normal fibres and in the sham-transfected compared to sham-normal fibres (figure 3.3).

This suggests possible individual differences in the cross-sectional area in MRF4 treated muscles and sham treated muscle possible due to differences in stretch and the area were the muscles were sectioned. The cross-sectional area in MRF4- and sham-transfected fibres was therefore also analysed as percentage of the normal fibres;

There is no significant difference in the cross-sectional area between the MRF4- and sham-transfected 2x and 2b fibres

There is a significant higher cross-sectional area in 2a fibres in the sham-transfected fibres compared to the MRF4-tansfected fibres but this group has very few observations (figure 3.4).

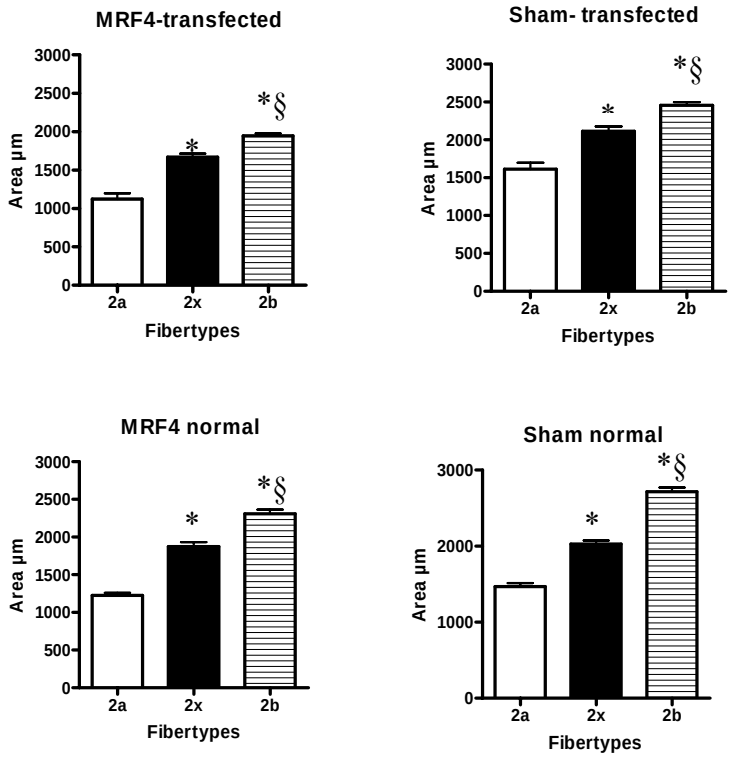


Figure 3.3 Cross-sectional area of each fibretype in normal fibres.
2a fibres have the lowest cross-sectional area, 2b fibres have the highest cross-sectional area and 2x fibres have intermediate cross-sectional area, this coincide with the general conception of the cross-sectional area in the different fibre types. The difference in the cross-sectional area between the fibre types is significant between 2a and 2x fibres, and 2a and 2b fibres (*), and between 2x and 2b fibres (§) in all experiment groups. The average area of the 2a, 2x and 2b fibre types show a significant difference in the cross-sectional area between the MRF4- and sham-transfected fibres and between the MRF4-normal fibres and sham-normal fibres, but not between the MRF4-transfected fibres and MRF4-normal fibres, and between sham-transfected and sham-normal fibres.

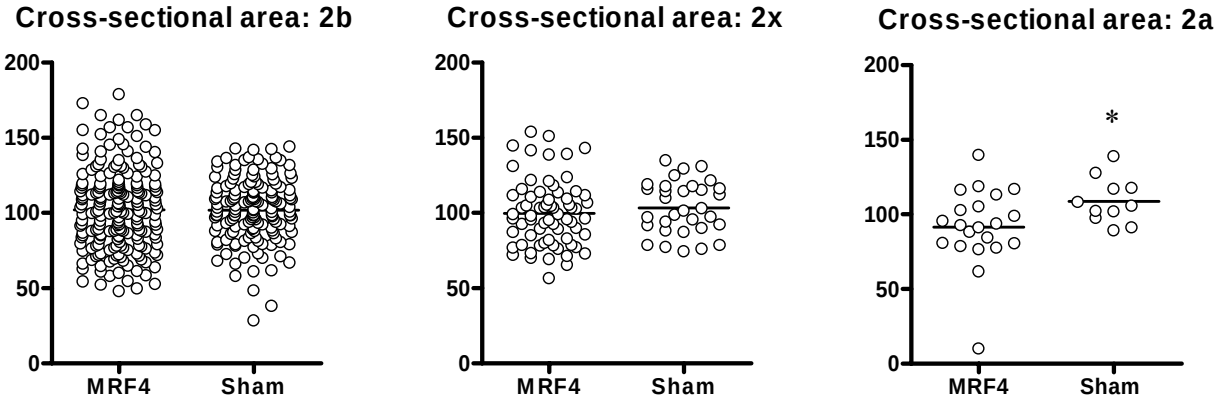


Figure 3.4 Cross-sectional areas in MRF4 and Sham transfected fibres.
The cross-sectional area as percentage of the average means of the normal fibres, show no significant difference between MRF4 -and sham-transfected 2b and 2x fibres. * There is a significant higher cross-sectional area in the sham-transfected 2a fibres compared to the MRF4-transfected 2a fibres, but there are very few observations in this group.

3.4. SDH activity

Normal SDH activity between fibre types show highest activity in the oxidative-glycolytic 2a fibres, lowest activity in the glycolytic 2b fibres and intermediate SDH activity in the 2x fibres. All experiment groups showed normal distribution of SDH activity between the fibre types (figure 3.5).

The average SDH activity in all muscles taken together, show a significant higher SDH activity in sham-transfected 2a fibres compared to MRF4 transfected 2a fibres and MRF4-normal 2a fibres, but not to sham-normal 2a fibres. There is no significant difference in SDH activity between MRF4-transfected 2a fibres and MRF4 normal fibres. There is a significant higher SDH activity in sham-transfected 2x fibres and sham-normal 2x fibres compared to MRF4-transfected 2x fibres. There is no significant difference in MRF4-transfected 2x fibres compared to MRF4-normal fibres, and MRF-normal 2x fibres compared sham-normal 2x fibres. There is no significant difference in SDH activity in 2b fibres between any of the experiment groups (figure 3.5).

These differences observed in SDH activity in 2a and 2x fibres, might due to either observations in some of the experiment groups or differences in the strength of SDH staining between the individual sections. To correct for these possible differences, the SDH activity was also analysed as percentage of the normal fibres.

There was no significant difference in the SDH activity between the MRF4 -and sham transfected 2a, 2x and 2b fibres (figure 3.6)

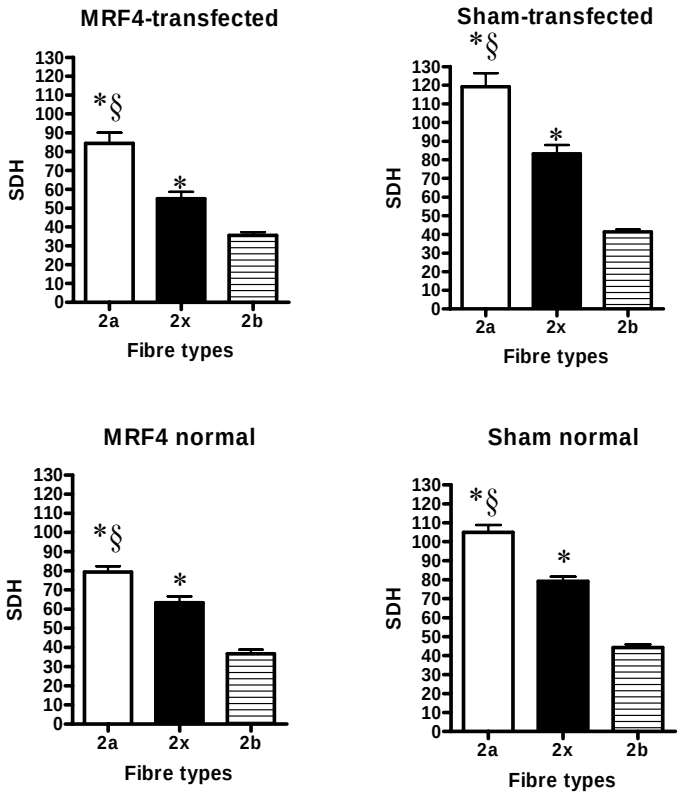


Figure 3.5 SDH activity in each fibre type.
There is a normal distribution of SDH activity between the fibre types in all experiment groups. 2a fibres have the highest SDH activity, 2b fibres have the lowest SDH activity and 2x fibres have intermediate activity. The oxidative capacity is significant higher in 2a fibres compared to 2x and 2b fibres (*), and in 2x fibres compared to 2b fibres (§) in all experiment groups. The average SDH activity in 2a fibres show a significant higher SDH activity in sham-transfected fibres compared to MRF4 transfected fibres and MRF4-normal fibres, but not compared to sham-normal fibres. There is no significant difference in MRF4-transfected 2a fibres and MRF4 normal 2a fibres. In 2x fibres there is significant higher SDH activity in sham-transfected fibres and sham-normal fibres compared to MRF4-transfected fibres. There is no significant difference in MRF4-transfected fibres compared to MRF4-normal fibres, and MRF-normal fibres and sham-normal fibres. There is no significant difference in the SDH activity in 2b fibres between the four experiment groups.

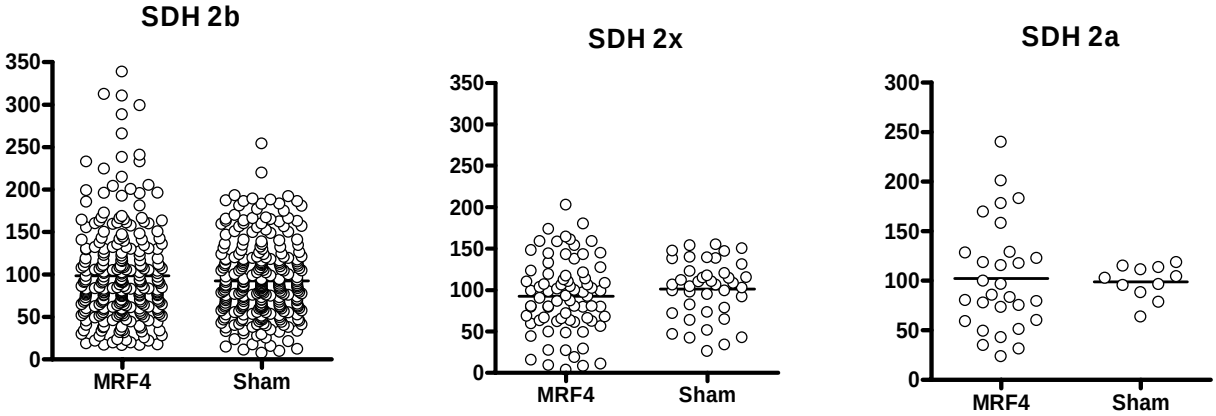


Figure 3.6 SDH activity in MRF4 -and Sham-transfected fibres.
The SDH activity as percentage of the average SDH activity in the normal fibres, show no significant difference in the SDH activity between MRF4 -and sham transfected 2a, 2x and 2b fibres.

4. Discussion

Overexpression of MRF4 did not lead to any change in the cross-sectional area and SDH activity compared to normal and sham-transfected fibres. The fibre type distribution showed a significant higher number of 2b fibres in both MRF4 -and sham-transfected fibres compared to normal, non-transfected fibres, and subsequent fewer 2a and 2x fibres in both MRF4 -and sham-transfected fibres, compared to non-transfected fibres. Because of a possible selective transfection of the largest fibres, it is problematic to determine whether there has been an effect of the MRF4 treatment on the MyHC phenotype.

4.1. Does MRF4 have an effect on MyHC expression?

When fast or slow muscles are subjected to a slow or fast electrical activity pattern, respectively, the activity pattern can induce a shift in the MyHC isoforms in a slow or fast direction (Lomo *et al.*, 1974; Eken & Gundersen, 1988; Ausoni *et al.*, 1990; Windisch *et al.*, 1998). This change in MyHC isoforms due to the electrical activity indicate that there are signalling pathways that link electrical activity to elements that regulate the expression of contractile proteins. In order to map these signalling pathways, several DNA regulatory sequences that might regulate the expression of contractile proteins has been studied, and regulatory elements that contain E- boxes and Mef-2 sites seems to be important in regulating the expression of contractile proteins. These regulatory sequences can bind to the myogenic bHLH factors, which suggest that at least some of these myogenic factors could be a link in the pathway between electrical activity and gene expression.

Hughes et al, (1997) saw that in mice absent of MyoD, there was a shift from a fast muscle fibre type toward a slower fibre type in fast muscles and a shift toward a faster phenotype in slow muscle. This suggests that MyoD has a role in maintaining the fast fibre type in adult muscle, and it's a further indication of that bHLH transcription factors might regulate the expression of MyHC isoforms in adult muscles.

Overexpression of MRF4 in the fast EDL muscle showed a significant higher number of 2b fibres in both MRF4 -and sham transfected fibres compared to non-transfected fibres. In addition there were also a significant higher number of 2b fibres in the sham-transfected fibres compared to MRF4-transfected fibres. If we just compare the MRF4-transfected fibres with the sham-transfected fibres, the smaller number of 2b fibres in the MRF-transfected fibres, might be

caused by a transformation in MyHC in the slow direction due to the treatment with MRF4, but if we compare the MRF-transfected fibres with the normal, non-transfected fibres there is no change in the slow direction, but rather in the fast direction. I propose that the higher amount of 2b fibres in MRF4-transfected fibres and sham-transfected fibres compared to non-transfected fibres is not a transformation of MyHC isoforms but I rather speculate that it is a result of a selective transfection as an effect of electroporation properties, effect of the lacZ reporter gene or both.

The transfection procedure might be more efficient in larger fibres apposed to smaller fibres as the conveyance of electrical current through the fibre might be facilitated in fibres with a larger cross-sectional area. There are technical aspects, like how you hold the electrodes, the distance between the electrodes, how much fluid that surrounds the muscle and how much voltage that is used, which all can influence the conveyance and effect of the current on the muscle. In this experiment, a combination of all these technical aspects and to low voltage might have an influence on the current so that it is only strong enough to induce a transfection in the largest fibres. Several studies have used this technique to study gene transfer *in vivo* (Ekmark *et al*., 2003, unpublished studies in our lab) where such properties of the electroporation has not been reported, it has however been reported in one similar study (Grönevik, master thesis, 2000), where a higher number of 2b fibres were observed in transfected fibres compared to normal fibres.

In addition to the possible selective transfection as an effect of the electroporation properties, there might be an effect of the lacZ itself. Vitadello et al. (1994) reported infiltrates of mononuclear cells surrounding about 50% of the fibre expressing lacZ. This indicates an immune reaction round the lacZ transfected cells and it can no be excluded that such an immune reaction might possible induce hypertrophy of fibres.

If one assumes a selective transfection of the largest fibres, then the difference in number of 2b fibres between the MRF4- and sham-transfected fibres, might be explained by a difference in fibre size in the individual muscles. The average area in sham-transfected fibres and sham-normal fibres are significant higher than in MRF4-transfected fibres and MRF4-normal fibres. This indicates that there is an individual difference in fibre size between the muscles probably due to difference in stretch and the position where the muscles were sectioned.

4.2. Has MRF4 an effect on the cross-sectional area?

The cross-sectional area is known to be smaller in type I fibres, increasing in 2a and 2x fibres, and be at its largest in 2b fibres. This arrangement is advantageous because slow enduring muscles need short diffusion distances to accommodate the high supply of oxygen and nutrition to the exterior.

Endurance training has been shown to induce a reduction in the cross-sectional area (Brown *et al.*, 1976; Tamaki, 1987), and overexpression of myogenin in the fast EDL muscle has also shown a reduction in the cross-sectional area of type II fibres, without a prior change in MyHC isoforms (Ekmark *et al.*, 2003)

Overexpression of MRF4 showed no change in the cross-sectional area in the MRF4-transfected fibres compared to the control group. Because of individual differences in the average area between the muscle pairs in each animal, the cross-sectional area was analysed as the percentage of the average cross-sectional area in the normal fibres. The average cross-sectional area in each fibre type in all experiment groups, show a normal arrangement of the cross-sectional area, where the 2a fibres have the smallest cross-sectional area and the 2b fibres have the largest cross-sectional area.

This suggests that MRF4 does not contribute in regulation of muscle fibre size in adult animals.

4.3. Has MRF4 an effect on the oxidative metabolism?

Increase in oxidative metabolism has been seen in many studies as a response to endurance training (Baldwin *et al.*, 1972; Holloszy & Booth, 1976). Ekmark et al, (2003) showed that overexpression of myogenin in the fast EDL muscle also induces an increased oxidative metabolism similar to the one that is seen during exercise. This change has been seen without a change in MyHC isoforms.

Overexpression of MRF4 showed no change in the SDH activity in MRF4-transfected fibres compared to any of the control groups. Amongst the type 2 fibre type population in normal fibres, the 2a fibres has the highest oxidative metabolism, while the 2b fibres are glycolytic and has the lowest oxidative metabolism. The average SDH activity in the different fibre types in this study also shows this normal pattern, and suggests that MRF4 does not have an effect on the oxidative metabolism in adult muscle fibres.

4.4. Does MRF4 have no role in regulating fibre type specific patterns of gene expression?

Zhang et al, (1995) also explored the idea of MRF4 being involved in fibre type specificity in adult muscles. They generated MRF4-null mice and looked at the expression of a subset of muscle specific genes. They saw no difference in transcripts of the contractile proteins; muscle creatine kinase (MCK), slow troponin I (TnI), a-skeletal actin and the β-subunit of the acetylcholine reseptor (AChR) in adult MRF4-null transgenes, compared to normal adult skeletal muscle. MyHCIIa, MyHCIIb and MyHCIIx were also expressed at normal levels in MRF4-null adults. The distribution of fast- and slow twitch fibres in adult MRF4-null hind limb muscle were also normal, and this lead to the conclusions that MRF4 is not required for expression of muscle specific genes at adult stages, and that MRF4 is not required to establish normal distribution of adult fast- and slow-twitch fibres. They also saw an up-regulation of myogenin in the MRF4 knock outs, which suggests that myogenin might compensate for the lack of MRF4 and that this compensation might have led to a normal expression of contractile proteins and a normal fibre type distribution in adult MRF4 knock outs. Compensatory mechanisms have been seen in several knock out studies in transgenes where, during the development, one or more factors seem to be able to compensate for the loss of another. My study was conducted on normal adult muscle to exclude or at least minimize such a compensatory mechanism (one can not exclude the existence of compensatory mechanisms in adult muscle as well). My findings in this study thereby further support the notion that MRF4 does not contribute to fibre type specificity in adult muscle.

Hughes et al. (1993) found that there is a selective accumulation of MyoD and myogenin mRNA in fast and slow adult skeletal muscle respectively, which suggests that the myogenic HLH transcription factors restrict expression of muscle genes to specific fibre types. They further suggest that individual genes may respond differently to distinct members of the myogenic HLH family, and this might explain how levels of MyoD and myogenin protein might control fibre type specific gene expression even though MRF4 mRNA is accumulated to much higher levels than either MyoD or myogenin mRNA in adult muscle fibres, and that it is possible that the myogenic HLH proteins discriminate between muscle-specific enhancers *in vivo*, perhaps through interaction with distinct coregulators.

This study and the study by Zhang et al contradicts the study by Voytik et al.(1993), which suggest that MRF4 plays a crucial role in preserving adult muscle fibre type based on the high levels of MRF4 mRNA that is expressed in adult muscle. Moreover, Weis et al. (2000) showed that despite the high level of MRF4 mRNA expression in adult muscle, they found no observations of MRF4 protein expression in normal innervated adult muscles using an antiserum specific to MRF4. They could however not exclude the possibility that MRF4 protein in innervated muscle is either expressed in so low levels or distributed throughout the cytoplasm to make it undetectable immunohistochemically, but this study might indicate that the protein expression does not coincide with the mRNA expression.

Walters et al, (2000) saw that MRF4 mRNA seems to be preferentially expressed in slow fibre types than in the fast fibre type population in the mixed gastrocnemius muscle and therefore suggest that MRF4 might exhibit a high degree of fibre type specificity at the mRNA level. They saw however no difference in expression between subpopulations of fibres in the slow soleus muscle and the mean levels of expression of MRF4 transcripts in type I fibres in the soleus were lower than in the type I fibres in the gastrocnemius muscle. This difference in MRF4 mRNA levels between the type I fibres in the two different muscles is suggested to due to different development pathways or neural activity patterns in the different muscle types.

Looking at all these studies and my study taken together, I think, that the role of MRF4 in preserving fibre phenotypes in adult muscle is certainly questioned and that the evidence leans towards suggesting that MRF4 does not have a role in regulating fibre type specific patterns of muscle gene expression in adult skeletal muscle.

4.5. Other possible functions of MRF4?

Supposed that MRF4 does not have a function in regulating fibre type specificity in adult skeletal muscle, what might the role of MRF4 be in adult muscles?

Looking further at the study by Weis et al. (2000), they, in addition to finding no MRF4 protein expression in innervated normal muscles, also investigated the MRF4 protein expression in denervated adult muscles. Here, they saw a quickly increase in nuclear MRF4 immunoreactivity after denervation. No immunostaining was detected beyond 14 days after denervation. There was no apparent difference in the expression of MRF4 protein between type I and type II fibres after the denervation which is consistent with the equal distribution of MRF4

mRNA transcripts in innervated and denervated slow-and fast-twitch muscle of rats. They also showed that MRF4 immunoreactivity was detected before MyoD and myogenin, and suggested that this faster appearance of protein might due to the already high levels of MRF4 mRNA. Their study suggests that MRF4 has a role in the transcriptional regulation of muscle genes as a response to denervation. Denervation causes a situation in the muscle similar to what occurs when a muscle is regenerating after damage, and Zhou & Bornemann (2001) showed that MRF4 is translated *in vivo* after a muscle have been stimulated to regenerate. They saw no expression of MRF4 protein in adult myoblasts, which differ from the other myogenin bHLH factors, but it was detected early in myofibre nuclei. They suggest that the MRF4 protein is not involved in the early regenerative events of proliferation and regeneration of adult myoblasts, but around and after the time of fusion into myofibres.

These studies suggest that MRF4 might have a role in the regeneration program in adult muscles after muscle damage.

4.6. Future perspectives

Based on the study by Weis et al. (2000), it would be interesting to further investigate the role of MRF4 after denervation. I did some experiments in this study where I overexpressed MRF4 in the EDL and then denervated the muscle to see if MRF4 could prevent transformation in a fast to slow direction that normally occurs after denervation (Huey & Bodine, 1998), but there were not enough data to give an indication of this and this ought to be explored further.

It could also be interesting to overexpress MRF4 in the mixed gastocnemius muscle, since there is evidence that MRF4 is expressed predominantly in the slow fibres in this muscle (Walters *et al.*, 2000).

The study by Zhou & Bornemann (2001) suggest further studies on regenerating muscle in MRF4-null mutants to clarify the role of MRF4.

4.7. Conclusions

- Overexpression of MRF4 in the fast EDL muscle gave no significant changes in the cross-sectional area and the SDH activity in type 2x and 2b fibres in MRF4-transfected fibres compared to sham-transfected fibres
- Overexpression of MRF4 in the fast EDL muscle gave a significant higher number of 2b fibres in both MRF4- and sham-transfected fibres compared to normal fibres and a subsequent decrease in the number of 2a and 2x fibres. I attribute this higher number of 2b fibres to be caused by a selective transfection of the largest fibres as an effect of electroporation properties, and not an effect of the treatment with MRF4.

These findings give no support to the hypothesis that MRF4 may have a role in regulating fibre type specific patterns of muscle gene expression in adult skeletal muscle.

5. Appendix A

5.1. Equethesin (catalognumber 702821, Ullevål farmasy)

Chloral hydrate	42,5 mg
Magnesium sulphate	21,0 mg
Pentobarbiton	9,7 mg
Ethanol (96%9	76,0 mg
Propylenglycol	428,0 mg
dH_2O	50,0 ml

5.2. pAP- MRF4 DNA solution (100µl)

pAP-MRF4 in H_2O (2µg/µl)	25 µl
NaCl, 4M	4µl
dH_2O	71 µl

5.3. pAP-MRF4 and pAP-lacZ DNA solution (100µl)

pAP-MRF4 in H_2O (2µg/µl)	25 µl
pAP-lacZ in H_2O (2µg/µl)	25 µl
NaCl, 4M	4µl
dH_2O	46 µl

5.4. Staining for β-gal activity

1. Wash the sections in PBS (pH=7,1)
2. Make the fixation solution:

Formaldehyde	2.0 g
Glutaraldehyde	400µl
10xPBS	10.0 ml
dH_2O	69.2 ml

Solve the formalaldehyde in dH_2O warmed to 60^0C. Adjust the volume to100 ml.

Adjust the pH to 7.4.

3. Fixate the sections at 4^0C
4. Wash the sections in PBS.
5. Make the staining solution:

10xPBS	150µl
Potassium Ferrocyanide	30µl
Potassium Ferricyanide	30µl
$MgCl_2$	3µl
dH_2O	1260µl
X-gal DMSO	30µl

6. Stain over night at 37^0C.
7. Wash the sections in PBS
8. Mount the sections in glycerine gel.

Gelatine	15.0 g
Glycerol	100 ml
dH_2O	100 ml

5.5. Staining for SDH activity

1. Make the tetrazolium solution:

MTT: 1mg/ml dH_2O	2,5 ml
Tris-buffer: 32,5 ml 0,1 M HCl in 42,5 dH_2O (pH 7.4)	2,5 ml
$CoCl_2$, 0.5M	0,5 ml
$MgCl_2$, 0.05M	1,0 ml
dH_2O	2,5 ml

2. Make the substrate solution:

Sodium succinate	6,75 g
dH_2O	8,0 ml

3. Make the staining solution:

Tetrazolium solution	0,9 ml
Substrate solution	0,1 ml

4. Make a hydrophobic ring round the sections with a hydrofobic-penn (H-4000, Vector).
5. Incubate the sections in the staining solution for 45 min at 37^0C.
6. Make fixation solution as in appendix 5.5.
7. Incubate the sections in the fixation solution for 20 min at 4^0C.
8. Wash the sections 3X5 min in PBS (pH 7.4).
9. Mount the sections in glycerine gel as in appendix 5.4.

5.6. Staining of MyHC

Staining for MyHC I, MyHC IIa, and *all* MyHC II antibodies.

1. Make a hydrophobic ring round the sections with a hydrofobic-penn (H-4000, Vector).
2. Dilute the primary antibody 1:1000 in 1% BSA in PBS (pH 7.4).
3. Incubate the sections with the primary antibody for 1 hour in room temperature.
4. Wash the sections 3X5 min in PBS (pH 7.4).
5. Dilute the secondary antibody 1.100 in 0,5% BSA in PBS.
6. Incubate the sections with the secondary antibody for 30 min at 37^0C.
7. Wash the sections 3X5 min in PBS (pH 7.4).

MyHC	Primary antibody	Secondary antibody
I	Sc-71	Anti-Mouse Ig, Rhodamine (1214608, Boeheringer)
IIa	MY-32	Anti-Mouse Ig, Rhodamine (1214608, Boeheringer)
All type II	BA-D5	Anti-Mouse Ig, Rhodamine (1214608, Boeheringer)

Staining for the MyHC IIb antibody

1. Make a hydrophobic ring round the sections with a hydrofobic-penn (H-4000, Vector).
2. Pre-incubate the sections with 1% PBS for 60 min at 4^0C.
2. Dilute the primary antibody 1:2000 in 0,5% BSA in PBS (pH 7.4).
3. Incubate the sections with the primary antibody for 45 min. in room temperature.
4. Wash the sections 3X5 min. in PBS (pH 7.4).
5. Dilute the secondary antibody 1.300 in 0,5% BSA in PBS.
6. Incubate the sections with the secondary antibody for 45 min. at 37^0C.
7. Wash the sections 3X5 min. in PBS (pH 7.4).

MyHC	Primary antibody	Secondary antibody
IIb	BF-F3	Anti-mouse IgM, Cy-3 (J115-165-020, Jackson ImmunoResearch Laboratories)

6. Appendix B

6.1. Abbreviations

AchR: Acetylcholine reseptor

ATP: Adenosin tri phosphate

β-gal: β-galactosidase

bHLH: Basic Helix Loop Helix

CaMK: Ca2+/Calmodulin dependent protein kinase

DNA: Deoxyribonuclei acid

EDL: Extensor digitorum longus

E8: Embryonic day eight

FFA: free fatty acid

MAPK:Mitogen activated protein kinase

MCK: Muscle creatin kinase

MDF: Myogenic determination factor

Mef-2: Myosite enhancer factor

MRF4: Myogenic regulation factor 4

mRNA: Messenger Ribonuclei acid

Myf-5: Myosin factor 5

MyHC: Myosineheavy chain

NFAT: Nuclear factor of activated t-cells

NIH: National institutes of health

PKC: Protein kinase C

PPARδ: Peroxisome proliferators activated receptor delta

PGC-1: Peroxisome proliferators activated receptor gamma coactivator 1

Ras: Rat sarcoma

RSV: Rouse sarcoma virus

SDH: Succinat dehydrogenase

SIT: silicon intensified tube

TnI: Troponin I

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